Vinculin is a major platelet protein that undergoes Ca^{2+} -dependent tyrosine phosphorylation

Jaroslav G. VOSTAL*† and N. Raphael SHULMAN†

*Division of Hematology, Center for Biologics Evaluation and Research, Food and Drug Administration, Bethesda, MD 20892, and †Clinical Hematology Branch, National Institutes of Diabetes, Digestive and Kidney Disease, Bethesda, MD 20892, U.S.A.

When intracellular Ca^{2+} pools are released during platelet stimulation by thrombin, elevation of platelet cytosolic Ca^{2+} concentration induces tyrosine phosphorylation of a 130 kDa protein, and refilling the pools mediates dephosphorylation of this protein [Vostal, Jackson and Shulman (1991) *J. Biol. Chem.* **266**, 16911–16916]. In the present work the 130 kDa protein was identified as vinculin by the following criteria. (1) It is detected on protein immunoblots of thrombin-activated platelets by both monoclonal anti-phosphotyrosine and anti-vinculin antibodies. (2) Removal of N-linked sugars with peptide-N-glycosidase or reduction did not change the molecular mass of vinculin or of the

130 kDa protein on SDS/PAGE. (3) The 130 kDa tyrosine-phosphorylated protein associates with Triton-soluble fraction of platelets as does vinculin. (4) The 130 kDa protein immunoprecipitated by anti-vinculin monoclonal antibody reacts with anti-phosphotyrosine antibody; when immunoprecipitated by anti-phosphotyrosine antibody it reacts with anti-vinculin antibody. (5) The 130 kDa tyrosine-phosphorylated protein and vinculin focus isoelectrically at pI 5.4–5.8. Our finding that vinculin is a major platelet protein that undergoes Ca^{2+} -dependent tyrosine phosphorylation during platelet activation may provide clues to the function of this protein.

INTRODUCTION

Platelet activation is associated with tyrosine phosphorylation of a number of proteins [1,2], the identity and function of which are not known. Tyrosine phosphorylation of subsets of proteins occurs along with platelet secretion [3] and aggregation [4,5], and inhibition of tyrosine phosphorylation is associated with decreases in responses of platelets to activating agonists [6,7]. Such observations suggest that tyrosine phosphorylation of specific proteins may regulate platelet activation. Further understanding of the role(s) of protein tyrosine phosphorylation lies in characterizing the substrates of, and the conditions which promote, tyrosine phosphorylation.

We have recently shown that tyrosine phosphorylation of three proteins with molecular masses of 130, 80 and 60 kDa is dependent on elevation of cytosolic Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$), as occurs after platelet activation with thrombin or other strong platelet agonists [8]. This phosphorylation appears to be due to activation of a cytoplasmic tyrosine kinase by the elevated $[\text{Ca}^{2+}]_i$, as well as to inhibition of tyrosine phosphatase activity by depletion of Ca^{2+} stores [8]. Because the 130 kDa protein was consistently the most intensely tyrosine-phosphorylated protein during platelet activation, we focused on determining its identity. Utilizing monoclonal antibodies against known platelet proteins of approx. 130 kDa which contain potential tyrosine-phosphorylation sites, we found that vinculin and the 130 kDa tyrosine-phosphorylated protein were identical in the physical characteristics of molecular mass, solubility in non-ionic detergent, lack of N-linked glycosylation and pI. Both proteins were immunoprecipitated with monoclonal anti-vinculin and anti-phosphotyrosine antibodies. These, and our previous observations that elevation of $[\text{Ca}^{2+}]_i$ induces this tyrosine phosphorylation [8], indicate that vinculin is tyrosine-phosphorylated in a Ca^{2+} -dependent manner during platelet activation. These observations were initially presented at the 1992 FASEB meeting [8a].

MATERIALS AND METHODS

Monoclonal antibodies

Monoclonal anti-phosphotyrosine antibody (clone 4G10) was obtained from Upstate Biotechnology Inc. (Lake Placid, NY, U.S.A.) and used at 1 $\mu\text{g}/\text{ml}$ for protein immunoblot development. Monoclonal anti-vinculin antibody (clone V284) was obtained from Boehringer Mannheim (Indianapolis, IN, U.S.A.) and used at 0.25 $\mu\text{g}/\text{ml}$ in protein immunoblots. A second monoclonal anti-vinculin antibody (clone VIN-11-5) was obtained from ICN Biomedicals (Irvine, CA, U.S.A.). Peptide-N-glycosidase (pNGase) was obtained from Oxford GlycoSystems (Rosedale, NY, U.S.A.). Monoclonal antibody to glycoprotein IIb was obtained from AMAC (Westbrook, ME, U.S.A.) and monoclonal antibody to CD 31 was obtained from Caltag (San Francisco, CA, U.S.A.).

Platelet isolation

Platelet-rich plasma was obtained by apheresis from volunteer donors, and incubated with indomethacin (10 μM) for 15 min at room temperature, and the platelets were gel-filtered into Tyrode/Hepes buffer (140 mM NaCl, 2.7 mM KCl, 12 mM NaHCO_3 , 0.4 mM NaH_2PO_4 , 0.5 mM MgCl_2 , 5.5 mM dextrose, 5 mM Hepes, pH 7.4) as described previously [8]. Platelets, adjusted to 400 000/ μl , were warmed to 37 °C for 15 min, and 10 ml of suspension was activated with thrombin (2.0 units/ml) for 3 min in the presence of 3 mM EGTA. The reaction was terminated by adding 10 ml of solubilization buffer (CHAPS 2.0%, NaCl 50 mM, NaF 50 mM, EDTA 5 mM, Hepes 5 mM, Na_2VO_4 2 mM, leupeptin 10 $\mu\text{g}/\text{ml}$, benzamidin 50 mM, phenylmethanesulphonyl fluoride 1 mM, pH 7.2). After incubation at room temperature for 30 min, the lysate was used in immunoprecipitations and in isoelectric-focusing procedures. Activation by thrombin and solubilization of platelets for protein immunoblot procedures in reduced and non-reduced buffer were

Abbreviations used: $[\text{Ca}^{2+}]_i$, intracellular free Ca^{2+} concentration; pNGase, peptide-N-glycosidase.

† To whom correspondence should be addressed.

done as described previously [8]. Thrombin activation was terminated by adding the platelet and thrombin mixture to an equal volume of pre-heated (90 °C) SDS/PAGE sample buffer (Tris/HCl 125 mM, SDS 0.4%, glycerol 20%, pH 8.5) and boiling for 3 min. Each lane of SDS/PAGE minigels (acrylamide; 7.5% Bio-Rad, Richmond, CA, U.S.A.) was loaded with 45–50 µg of protein, which was equivalent to approx. 24×10^6 platelets. Protein immunoblots illustrated are representative of results obtained from at least three separate experiments unless otherwise stated. Densitometry on protein immunoblots was performed with GS 3000 scanning densitometer (Hoeffer Scientific Instruments, San Francisco, CA, U.S.A.).

N-linked sugar removal

N-linked sugars were enzymically removed with pNGase according to the manufacturer's instructions. pNGase (4 units, 20 µl) was incubated with 100 µg (100 µl) of lysate of thrombin-activated platelets after denaturation by heating at 100 °C for 2 min. Incubation was terminated after 24 h at room temperature by addition of an equal volume of SDS/PAGE buffer and boiling for 3 min. A 40 µl portion of this mixture was separated on SDS/PAGE, transblotted and developed with respective monoclonal antibodies.

Immunoprecipitation

A 250 µl portion of lysate from thrombin-activated platelets was incubated with 50 µl of Protein G-agarose (Sigma) for 4 h at 4 °C to clear the lysate of non-specifically binding proteins. Lysate and Protein G-agarose were then separated by centrifugation at 10000 *g* for 1 min. The supernatant was incubated for 16 h at 4 °C with a fresh 50 µl of Protein G-agarose and 2 µg (20 µl) of anti-vinculin antibody or with 2 µg (20 µl) of anti-phosphotyrosine antibody. The Protein G-agarose and antibody complex were separated by centrifugation as above, washed three times with solubilization buffer and mixed with 100 µl of SDS/PAGE sample buffer diluted 1:1 with water. Samples were separated on SDS/PAGE and transblotted as described above. Immunoprecipitates obtained with anti-phosphotyrosine antibody were run under reducing conditions, since the non-reduced anti-phosphotyrosine antibody migrated at 120 kDa and could be confused with the 130 kDa protein, but reduced antibody migrated as 50 kDa and 25 kDa polypeptides. Reduction did not change migration of either vinculin or the 130 kDa tyrosine-phosphorylated protein. For sequential immunoprecipitation of the same supernatant, each cycle was started by addition of 50 µl of Protein G-agarose and 2 µg of anti-vinculin antibody, and mixtures were incubated for 16 h each at 4 °C. The final supernatant was mixed 1:1 with SDS/PAGE sample buffer and analysed as described above.

Isoelectric focusing

A 20 ml portion of solubilized platelets was dialysed overnight at 4 °C against 4 litres of deionized water. The volume of the dialysed sample was adjusted to 50 ml with deionized water, and CHAPS at 2% final concentration was added to resuspend precipitated proteins. Ampholytes (pH range 4–6 and 6–8) were added to a final concentration of 2%, and the sample was focused on a Rotofor isoelectric-focusing apparatus (Bio-Rad) according to the manufacturer's instructions. Twenty fractions (each approx. 2 ml) were collected; and, on the basis of pH determinations, the sample was refocused if necessary to obtain a smooth pH gradient. Sodium vanadate (100 µM final concn.) was added to each of the 20 fractions to inhibit dephosphorylation

during their 10-fold concentration on Centricon micro-concentrators (Amicon, Beverly, MA, U.S.A.) with 30 kDa molecular-mass cut off. Fractions were mixed with an equal volume (200 µl) of SDS/PAGE sample buffer and boiled for 3 min. Then 50 µl of buffer containing 30–40 µg of protein was loaded per lane, and proteins were separated on SDS/PAGE minigels (7.5% acrylamide) and transblotted.

Distribution of the 130 kDa protein in Triton X-100-treated platelets

A 1 ml portion of gel-filtered platelets at 10^6 cells/µl in Tyrode buffer containing 3 mM EGTA were activated with 2 units of thrombin/ml at 37 °C for 3 min. Then 100 µl of solubilization buffer (final concns.: Triton X-100 1%, NaF 50 mM, Hepes 20 mM, Na_3VO_4 1 mM, phenylmethanesulphonyl fluoride 1 mM, leupeptin 10 µg/ml, pH 7.2) was added to the platelets, and the solubilized cells were placed on ice for 20 minutes. Lysates were centrifuged at 12000 *g* for 5 min. The resultant pellets were washed with solubilization buffer, re-centrifuged, solubilized with 100 µl of SDS/PAGE buffer and boiled for 3 min. Supernatants were concentrated 10-fold on Centricon micro-concentrators with 30 kDa molecular-mass cut off, mixed with an equal volume of SDS/PAGE buffer and boiled for 3 min. Lanes on SDS/PAGE gels were loaded with Triton-soluble proteins derived from 15×10^6 cells or with Triton-insoluble proteins from 75×10^6 cells.

RESULTS

Glycoprotein IIb, CD31 and vinculin all have similar molecular masses in the range of 125–140 kDa on SDS/PAGE, and each contains potential tyrosine-phosphorylation sites [9–11]. After removal of N-linked sugars from proteins in platelet lysates and separation of proteins by SDS/PAGE, transblots of the gels were probed with monoclonal antibodies against the respective proteins (Figure 1). The electrophoretic mobility of the tyrosine-phosphorylated 130 kDa protein and vinculin did not change after treatment with pNGase, whereas the apparent molecular mass of both glycoprotein IIb and CD31 was decreased. When

Figure 1 Effect of pNGase treatment of platelet lysates on the 130 kDa tyrosine-phosphorylated protein, glycoprotein IIb, CD-31 and vinculin

Platelet lysates were separated by SDS/PAGE after treatment with pNGase to remove N-linked sugars and compared with control lysates (c). Protein immunoblots were developed with anti-phosphotyrosine (anti-PY), anti-glycoprotein IIb (anti-IIb), anti-CD-31 and anti-vinculin (anti-vinc) antibodies. Arrow indicates the 130 kDa protein. Molecular masses of protein standards are indicated by kDa in this and other Figures.

Figure 2 Protein immunoblots of Triton-insoluble and Triton-soluble fraction from thrombin-activated platelets developed with anti-phosphotyrosine (anti-PY) and anti-vinculin (anti-vinc) antibodies

Cytoskeletal pellets and supernatants were prepared as described in the Materials and methods section. The Triton-insoluble protein (insol.) loaded per lane was obtained from 75×10^6 cells and Triton-soluble protein (sol.) was obtained from 15×10^6 cells. Arrow indicates the 130 kDa protein.

platelet lysates were separated under reduced conditions on SDS/PAGE, glycoproteins IIb and CD31 had lower mobility, whereas that for vinculin or the 130 kDa tyrosine-phosphorylated protein did not change (results not shown). These observations

Figure 4 Sequential immunoprecipitation of platelet lysate with anti-vinculin antibody

Thrombin-activated platelet lysates were precipitated with three cycles of anti-vinculin and Protein G-agarose. Protein immunoblots of the first and third immunoprecipitate pellets and the third (final) supernatants were developed with anti-vinculin (anti-vinc; **a**) or anti-phosphotyrosine (anti-PY; **b**) antibody. Abbreviations: lys, platelet lysate; ppt, immunoprecipitate; sup, supernatant. Arrow indicates the 130 kDa protein.

eliminated glycoprotein IIb or CD-31 as the 130 kDa tyrosine-phosphorylated protein.

Figure 2 shows that the 130 kDa protein identified by anti-phosphotyrosine antibody is detectable only in the Triton-soluble fraction of thrombin-activated platelets, and that almost all of the protein identified by anti-vinculin antibody is found in the same fraction. The trace amount of material identified by anti-vinculin antibody in the Triton-insoluble fraction may reflect the possible greater sensitivity of the anti-vinculin compared with the

Figure 3 Detection of 130 kDa tyrosine-phosphorylated protein in anti-vinculin immunoprecipitates of thrombin-activated platelet lysate

Lysates of activated platelets were incubated with Protein G-agarose for 4 h at 4 °C to pre-clear the lysate of non-specifically binding proteins and then were immunoprecipitated with anti-vinculin and Protein G-Sepharose overnight. The pellet was washed three times with lysis buffer, resuspended in 100 μ l of SDS/PAGE sample buffer, and the proteins were separated on SDS/PAGE. Gels were transblotted and protein immunoblots were developed with anti-vinculin (**a**) or with anti-phosphotyrosine (**b** and **c**) antibodies. As a control, the precipitating anti-vinculin antibody was not present during the immunoprecipitation in the second lane of panel (**c**). Protein immunoblots of the anti-vinculin immunoprecipitates were also developed with anti-IIb and anti-CD31 antibodies (**d**). Abbreviations: lys, lysate of thrombin-activated platelets; ppt, immunoprecipitate; sup, supernatant; plts, platelets; anti-vinc, anti-vinculin; anti-PY, anti-phosphotyrosine; anti-IIb, anti-glycoprotein IIb; anti-CD31, anti-glycoprotein CD31. Arrow indicates the 130 kDa protein.

Figure 5 Detection of vinculin in anti-phosphotyrosine immunoprecipitates of thrombin-activated lysates

Lysates of activated platelets were immunoprecipitated as described in Figure 1 legend and the Materials and methods section. Protein immunoblots of the precipitates and lysates were developed with anti-vinculin or anti-phosphotyrosine (a) antibody. Anti-vinculin and anti-phosphotyrosine immunoprecipitates were developed with a separate anti-vinculin monoclonal antibody (ICN Biomedicals) (b). Abbreviations: lys, lysate of thrombin-activated platelets; ppt, immunoprecipitate; anti-vinc, anti-vinculin; anti-PY, anti-phosphotyrosine. Arrow indicates the 130 kDa protein.

anti-phosphotyrosine assay, or the possibility that tyrosine phosphorylation involves a subset of vinculin isoforms [12].

Anti-vinculin antibody immunoprecipitated vinculin, a 150 kDa precursor to vinculin, metavinculin, and vinculin degradation products with molecular masses of 95 and 85 kDa from thrombin-activated platelet lysates (Figure 3a). Anti-phosphotyrosine antibody reacted with immunoprecipitated vinculin on protein immunoblots, but not with its degradation products (Figure 3b). Possible non-specific interaction of the 130 kDa protein with Protein G-agarose was ruled out by finding that this phosphorylated protein was not detected when anti-vinculin antibody was left out of the immunoprecipitation mixture (Figure 3c) and that glycoproteins IIb and CD31 were not detected on immunoblots of the anti-vinculin immunoprecipitates (Figure 3d). Moreover, the lysate of thrombin-activated platelets could be depleted of the 130 kDa tyrosine-phosphorylated protein by repeated immunoprecipitation with anti-vinculin antibody (Figure 4). After three cycles of precipitation as described in the Materials and methods section, the supernatant contained 13% of the starting amount of vinculin and no detectable 130 kDa tyrosine-phosphorylated protein, as shown by densitometry measurements using anti-vinculin and anti-phosphotyrosine antibody respectively. Similarly, both vinculin and the 130 kDa protein were proportionately immunoprecipitated by anti-phosphotyrosine antibody (Figure 5a). To rule out a possible non-specific interaction of the vinculin antibody with the 130 kDa protein, we utilized a separate monoclonal anti-vinculin antibody (clone VIN-11-5) to probe protein immunoblots of the first anti-vinculin (clone V284) and anti-phosphotyrosine immunoprecipitates. Both anti-vinculin

Figure 6 Protein immunoblots of thrombin-activated platelet lysates isoelectrically focused and separated by SDS/PAGE

Lysates were focused in a preparative-scale isoelectric-focusing apparatus as described in the Materials and methods section, the fractions were concentrated, and proteins were separated on SDS/PAGE and transblotted. Duplicate sets of blots of fractions obtained at different pH values were developed separately with anti-vinculin or anti-phosphotyrosine antibody. The arrows indicate the 130 kDa protein detected by both antibodies.

antibodies reacted equally (Figure 5b). These findings can be interpreted variously to indicate that: (1) the 130 kDa protein is tyrosine-phosphorylated vinculin; (2) vinculin and the 130 kDa protein are both tyrosine-phosphorylated and have the same apparent molecular mass on SDS/PAGE; (3) a non-tyrosine-phosphorylated form of vinculin associates with the 130 kDa tyrosine-phosphorylated protein.

To resolve these possibilities, we performed preparative-scale isoelectric focusing on lysates of thrombin-activated platelets, electrophoresed resultant fractions on SDS/PAGE, and developed the protein immunoblots made therefrom with anti-vinculin and anti-phosphotyrosine antibodies. This method has an advantage over two-dimensional gels (isoelectric focusing: SDS/PAGE) in that larger quantities of protein can be separated for detection on protein immunoblots. Figure 6 shows that a 130 kDa protein which focuses at pI 5.4–5.8 reacts with both anti-vinculin and anti-phosphotyrosine antibodies. There was no other 130 kDa tyrosine-phosphorylated protein in fractions pH 5.2–9.0. The same pI for vinculin was also obtained on conventional polyacrylamide-flat-bed isoelectric focusing (results not shown).

DISCUSSION

Elevation of $[Ca^{2+}]_i$, whether induced by agonists, Ca^{2+} ionophore or thapsigargin, results in tyrosine phosphorylation of the same set of platelet proteins [8]. This observation suggests that tyrosine phosphorylation of these proteins participates in Ca^{2+} -dependent platelet activation. A 130 kDa protein detected by monoclonal (4G10 clone) anti-phosphotyrosine antibody on protein immunoblots is the most intensely phosphorylated of these proteins. We have identified this protein as vinculin on the basis of molecular mass, lack of disulphide bridges or N-linked glycosylation, characteristic distribution in non-ionic detergent, cross-reactivity of the protein with the opposite antibody immunoprecipitated by anti-vinculin or anti-phosphotyrosine, and a compatible pI of 5.4–5.8 for a single isoelectrically focused component that reacted with both anti-vinculin and anti-phosphotyrosine antibody.

The molecular mass of vinculin has been reported to be 117 kDa, on the basis of its amino acid sequence [13], but vinculin frequently is reported as 130 kDa protein on SDS/PAGE [14–16]. In resting platelets more than 99% of vinculin is cytosolic, and after thrombin activation approx. 10% associates with the cytoskeleton [17]. The 130 kDa protein distributes in the same proportion as vinculin in the Triton-soluble (cytosolic) and insoluble (cytoskeletal-associated) fractions of thrombin-activated platelets as detected by anti-vinculin, but was present only in the cytosolic fraction when anti-phosphotyrosine antibody was used. Since tyrosine-phosphorylated vinculin most likely accounts for only 1–2% of total vinculin [18], it may not be detectable in the relatively small amounts of vinculin (10% or less of total) that associates with the cytoskeleton. This is supported by the serial immunoprecipitation of thrombin-activated platelet lysates with anti-vinculin, where levels of less than 13% of the total vinculin were not detected by the anti-phosphotyrosine antibody.

The pI for vinculin determined on the Rotoform apparatus and by flat-bed isoelectric focusing was in the range 5.4–5.8, which is lower than the pI (6.3–6.6) reported for platelet vinculin in one other study [17]. These differences most likely are attributable to differences in techniques, but more important than the absolute pI for the present study is the reactivity of a single 130 kDa protein in the same isoelectrically focused fractions with both anti-vinculin and anti-phosphotyrosine antibodies.

Vinculin has been reported to exist in a phosphorylated state in resting platelets as measured by ^{32}P incorporation; but the specific amino acid residues phosphorylated were not identified [17], and no increase in total phosphorylation occurred, as shown by isotopic analysis, when platelets were activated with thrombin [17,19]. In chick fibroblasts 89% of ^{32}P incorporation into vinculin was found in phosphoserine, 9% was on phosphothreonine and only 2% on phosphotyrosine [11], suggesting that the reported ^{32}P incorporation into platelet vinculin was due primarily to phosphorylation of serine and threonine residues. Tyrosine phosphorylation of vinculin associated with platelet activation appears to account for 10% or less of total phosphorus incorporated into vinculin and would not be clearly detectable as an increase in total ^{32}P incorporation.

The function of vinculin in platelets and other cells is not defined. Vinculin has been postulated to connect plasma membranes with microtubules, since it is frequently localized at sites of intracellular focal adhesion [16], where the plasma membrane interacts with the cytoskeleton. Vinculin binds talin [20,21] and α -actinin [22], both of which can bind actin [23], suggesting that vinculin may be functionally integrated into the cytoskeleton. In platelets vinculin has been postulated to function in clot retraction, where it would serve as a cytoskeletal anchor for contracting actin microfilaments [24].

Tyrosine phosphorylation of vinculin has been detected in growth cones of neurons [25] and in normal fibroblasts [18], but the degree of tyrosine phosphorylation did not change during the normal cell cycle, indicating that such phosphorylation is not associated with a cell-cycle-specific step [26]. However, increased tyrosine phosphorylation of vinculin was found in most malignant transformed cells, but this phosphorylation was not sufficient to induce the transformation [18]. In transformed fibroblasts vinculin appears to be a substrate for the cytoplasmic tyrosine kinase pp60^{v-src}, the product of a transforming viral oncogene [11]. Platelets contain a number of *src* tyrosine kinases such as *fyn* and *lyn*, and a high concentration of pp60^{v-src} [27], which could phosphorylate vinculin in platelets. Recently, the activation of tyrosine kinases by elevation of $[Ca^{2+}]_i$ has been demonstrated in epithelial cells [28]. Whether elevated $[Ca^{2+}]_i$ directly or indirectly activates platelet tyrosine kinases to cause phosphorylation of vinculin remains an open question. Platelets provide a convenient normal cell that can be activated by a variety of agonists for further studies of the tyrosine kinase and phosphatase activities that appear to regulate tyrosine phosphorylation of vinculin.

REFERENCES

- 1 Ferrell, J. E., Jr. and Martin, G. S. (1988) *Mol. Cell. Biol.* **8**, 3603–3610
- 2 Golden, A. and Brugge, J. S. (1989) *Proc. Natl. Acad. Sci. U.S.A.* **86**, 901–905
- 3 Nakamura, S. and Yamamura, H. (1989) *J. Biol. Chem.* **264**, 7089–7091
- 4 Ferrell, J. E. and Martin, G. S. (1989) *Proc. Natl. Acad. Sci. U.S.A.* **86**, 2234–2238
- 5 Golden, A., Brugge, J. S. and Shattil, S. J. (1990) *J. Cell Biol.* **111**, 3117–3127
- 6 Salari, H., Duronio, V., Howard, S. L., Demos, M., Jones, K., Reany, A., Hudson, A. T. and Pelech, S. L. (1990) *FEBS Lett.* **263**, 104–108
- 7 Dhar, A., Paul, A. K. and Shukla, S. D. (1990) *Mol. Pharmacol.* **37**, 519–525
- 8 Vostal, J. G., Jackson, W. L. and Shulman, N. R. (1991) *J. Biol. Chem.* **266**, 16911–16916
- 8a Vostal, J. G. and Shulman, N. R. (1992) *FASEB J.* **6**, A1761 (abstr. 4776)
- 9 Findik, D., Reuter, C. and Presek, P. (1990) *FEBS Lett.* **262**, 1–4
- 10 Metzelaar, M. J., Korteweg, J., Sixma, J. J. and Niewenhuys, H. K. (1991) *Thromb. Haemostasis* **66**, 700–707
- 11 Sefton, B. M., Hunter, T., Ball, E. H. and Singer, S. J. (1981) *Cell* **24**, 165–174
- 12 Geiger, B. (1982) *J. Mol. Biol.* **159**, 685–701
- 13 Weller, P. A., Ogrzyzko, E. P., Corben, N. I., Zhidkova, B., Patel, N. K., Spurr, N. K., Kotliansky, V. E. and Critchley, D. R. (1990) *Proc. Natl. Acad. Sci. U.S.A.* **87**, 5667–5671
- 14 Geiger, B. (1979) *Cell* **18**, 193–205

- 15 Feramisco, J. R. and Burridge, K. (1990) *J. Biol. Chem.* **255**, 1194–1199
- 16 Burridge, K., Fath, K., Kelly, T., Nuckolls, G. and Turner, C. (1988) *Annu. Rev. Cell Biol.* **4**, 487–525
- 17 Bruin, T., Asijee, G. M., Prins, A., Wouter ten Cate, J. and Sturk, A. (1991) *Thromb. Haemostasis* **65**, 206–211
- 18 Kellie, S. (1988) *BioEssays* **8**, 25–30
- 19 Hagmann, J. and Burger, M. M. (1992) *J. Cell. Biochem.* **50**, 237–244
- 20 Burridge, K. and Magneat, P. (1984) *Nature (London)* **308**, 744–746
- 21 Jones, P., Jackson, P., Price, G. J., Patel, B., Ohanion, V., Lear, A. L. and Critchley, D. R. (1989) *J. Cell Biol.* **109**, 2917–2927
- 22 Wachstock, D. H., Wilkins, J. A. and Lin, S. (1987) *Biochem. Biophys. Res. Commun.* **146**, 554–560
- 23 Muguruma, M. S., Matsumura, S. and Fukazawa, T. (1990) *Biochem. Biophys. Res. Commun.* **171**, 1217–1223
- 24 Horvath, A. R. and Muszbeck, L. (1988) in *Structure and Function of the Cytoskeleton* (Rousset, B., ed.), pp. 127–132, Colloque INSERM/John Libbey Eurotext, Paris
- 25 Igarashi, M., Saito, S. and Komia, Y. (1990) *Eur. J. Biochem.* **193**, 551–558
- 26 Rosok, M. J. and Rohrschneider, L. R. (1983) *Mol. Cell. Biol.* **3**, 475–479
- 27 Golden, A. S., Nemeth, S. P. and Brugge, J. S. (1989) *Proc. Natl. Acad. Sci. U.S.A.* **83**, 852–856
- 28 Huckle, W. R., Dy, R. C. and Earp, H. S. (1992) *Proc. Natl. Acad. Sci. U.S.A.* **89**, 8837–8841